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Quantitative Aspects in the Assessment of Liver Injury

by Gabriel L. Plaa*

Liver function data are usually difficult to use in their original form when one wishes to compare the hepatotoxic properties of several chemical substances. However, procedures are available for the conversion of liver function data into quantal responses. These permit the elaboration of dose-response lines for the substances in question, the calculation of median effective doses and the statistical analysis of differences in liver-damaging potency. These same procedures can be utilized for estimating the relative hazard involved if one compares the liver-damaging potency to the median effective dose for some other pharmacologic parameter. Alterations in hepatic triglycerides, lipid peroxidation, and the activities of various hepatic enzymes can also be quantitated in a dose-related manner. This permits the selection of equitoxic doses required for certain comparative studies and the selection of doses in chemical interaction studies. The quantitative problems involved in low-frequency adverse reactions and the difficulty these present in the detection of liver injury in laboratory animals are discussed.

Conversion of Liver Function Data into Quantal Responses

In order to compare potencies of compounds it is necessary to establish dose–response parameters in terms which can be statistically analyzed. Such procedures are done routinely in the determination of the median lethal dose (LD_{50}), the dose which kills 50% of the population under test. The traditional manner is to plot along the ordinate the probit transformation of the percentage of animals that died and to plot the dose, or the logarithm of the dose, along the abscissa. Similar types of transformations can be carried out with biochemical parameters used for assessing hepatic function.

In 1958 it was demonstrated (*1*) that the prolongation of barbiturate sleeping time induced by liver injury could be used in such a manner. Figure 1 demonstrates the dose–response curves which were elaborated from such data for seven halogenated hydrocarbons with different hepatotoxic potencies. The conversion of these data into quantal responses is relatively simple. What one needs to do in the case of pentobarbital sleeping time is to establish the mean sleeping time in a large number of control animals. From this mean one can calculate the standard deviation and establish the limits of normalcy, usually a value two or three standard deviations removed from the mean control response. Any value in the test population which is larger than this upper limit of normalcy is then considered to be an abnormal response. In each group of animals subjected to a single dose of halogenated hydrocarbon one determines the number of animals exhibiting a normal or abnormal sleeping time. These quantal data are then converted to percentages and plotted as one normally plots lethality data. Once achieved, these data permit the comparison of the dose-response lines, tests for parallelism and statistical analyses of potency differences.

While the data in Figure 1 were derived by use of prolonged barbiturate sleeping time as the index of hepatic dysfunction, it was subsequently demonstrated that plasma sulfobromophthalein (BSP) retention data or elevation in serum transaminase (SGPT) data could be handled in a similar manner (*2–4*). The prime prerequisite was

* Département de Pharmacologie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada.

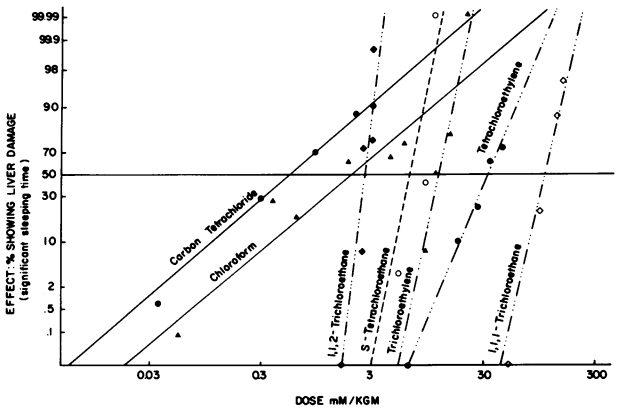

FIGURE 1. Dose-response curve for the effect of seven halogenated hydrocarbons on prolongation of pentobarbital sleeping time in mice. All hydrocarbons were given subcutaneously; pentobarbital sleeping time was determined 24 hr later. Redrawn from Plaa et al. (*1*) with permission of the Williams and Wilkins Co.

that a large control population be measured and that upper limits of normalcy be established.

Table 1 shows the median effective doses for lethality (LD_{50}), the BSP retention ED_{50}, and the SGPT elevation ED_{50}. It can be seen that the two parameters of hepatic dysfunction have ED_{50} values which are much smaller than the 24-hr LD_{50}, except for tetrachloroethylene. It should be mentioned that the 24-hr LD_{50} is actually a measure of the central nervous system depressing properties of the halogenated hydrocarbon and is not a measure of lethality due to hepatic dysfunction.

Table 2 shows that when one ranks the six halogenated hydrocarbons, according to their relative potencies then the 24-hr LD_{50} is a poor index of the hepatotoxic potencies of these compounds. Carbon tetrachloride appears to be one of the least potent halogenated hydrocarbons if one uses the LD_{50} as the parameter; however, if one uses BSP retention or SGPT elevation as the parameter, carbon tetrachloride becomes the most potent of this series. The relative potencies

Table 1. Median effective doses for lethality and parameters of hepatic dysfunction for six halogenated hydrocarbons in mice.[a]

Compound	Median effective dose, mmole/kg (IP)		
	24-hr LD_{50}	BSP retention ED_{50}	SGPT elevation ED_{50}
Carbon tetrachloride	28	0.6	0.1
Chloroform	14	9.7	2.3
1,1,1-Trichloroethane	37	27	2.5
1,1,2-Trichloroethane	3.7	2.7	1.1
Trichloroethylene	24	23	18
Tetrachloroethylene	28	32	28

[a] Data obtained from Klaassen and Plaa (*3*).

for BSP and SGPT agree quite well with the known relative hepatotoxic potencies of these halogenated hydrocarbon solvents based on industrial experience.

While comparison of relative potencies based on only one hepatotoxic parameter can be useful, other types of quantitative comparisons can be made to indicate the relative hazard involved with exposure to such substances. Table 3 depicts the potency ratios (LD_{50}/ED_{50}) for the six halogenated hydrocarbons in question, SGPT elevation being used as the parameter of hepatic dysfunction. It can be seen that carbon tetrachloride has a potency ratio which is quite large and quite different from those established for the other halogenated hydrocarbons. A potency ratio of 280 for carbon tetrachloride means that the effective dose for producing liver injury in mice is about 1/300 the dose required to produce death primarily by central nervous system depression. On the other hand, for tetrachloroethylene the potency ratio of 1.0 indicates that the dose which produces liver injury is essentially identical to the dose that produces death by central nervous system depression. Table 3 shows the same type of data generated from results using BSP retention as the parameter of dysfunction. One notices again that carbon tetrachloride has a much larger potency ratio than the other compounds. The dose producing liver injury is about 1/50 of the

Table 2. Relative potency rankings of six halogenated hydrocarbons in mice.[a]

24-hr LD_{50}	BSP retention ED_{50}	SGPT elevation ED_{50}
1,1,2-Trichloroethane	Carbon tetrachloride	Carbon tetrachloride
Chloroform	1,1,2-Trichloroethane	1,1,2-Trichloroethane
Trichloroethylene	Chloroform	Chloroform
Tetrachloroethylene	Trichloroethylene	1,1,1-Trichloroethane
Carbon tetrachloride	1,1,1-Trichloroethane	Trichloroethylene
1,1,1-Trichloroethane	Tetrachloroethane	Tetrachloroethane

[a] Data obtained from Klaassen and Plaa (*3*). Each list progresses downward from most potent to least potent hydrocarbon.

Table 3. Potency ratios of six halogenated hydrocarbons for SGPT elevation or BSP retention in mice.[a]

Compound	BSP-retention potency ratio (LD_{50}/ED_{50})	SGPT-elevation potency ratio (LD_{50}/ED_{50})
Carbon tetrachloride	45	280
Chloroform	1.5	6.4
1,1,2-Trichloroethane	1.4	3.4
1,1,1-Trichloroethane	1.4	1.5
Trichloroethylene	1.1	1.4
Tetrachloroethylene	0.9	1.0

[a] Calculated from data in Table 1. Data obtained from Klaassen and Plaa (*3*).

dose producing death within 24 hr. With trichloroethylene the dose required to produce liver injury is virtually identical with the dose required to produce death.

Potency ratios derived in this matter can be used to acquire a quantitative assessment of the relative hazard involved in exposures of these various agents. In an industrial environment it is relatively easy to determine whether the concentration of carbon tetrachloride in the atmosphere is sufficiently high to produce mild depression of the central nervous system (dizziness, ataxia, etc.). However it is quite difficult to determine whether the concentration in the atmosphere is sufficiently high to produce liver injury since this requires a matter of days before the signs of toxicity develop. If an industrial environment is controlled in such a manner that the concentration in the air being breathed by the individual is sufficiently low to prevent visible signs of central nervous system depression one has also controlled the environment against the liver damaging properties of a compound like trichloroethylene since the potency ratio for this compound is about 1. However with carbon tetrachloride, control of the environmental atmosphere for effects on the central nervous system only does not eliminate the possibility that the concentration is sufficiently high to produce liver injury, because of the large potency ratio for this compound. Therefore this type of experimental quantitative comparison of potency can give some indication of the relative hazard that may be involved with these substances in occupational environments.

It should be noted that the animals in these experiments received the halogenated hydrocarbons either by the subcutaneous or the intraperitoneal route of administration. However, workers exposed to these solvents usually inhale them. Therefore one can question whether data derived by the parenteral route of administration can be useful for estimating relative hazard of inhalation. From a theoretical standpoint the potency ratio derived by the parenteral route of administration should resemble that derived by the inhalation route, since the pharmacological effects are manifestations of the amount of substance that has been absorbed. However, it is known that the route of administration can affect the amount of substance absorbed and its distribution in tissues. Gehring (*5*) investigated this aspect and compared the relative potencies of a series of halogenated hydrocarbons when they were administered by parenteral route to those obtained when they were given by inhalation. He found that, while there were some differences in the absolute value of the potency ratio, the relative rankings of the halogenated hydrocarbons were quite similar with both routes of administration, indicating that even parenterally administered compounds can give an indication about the relative hazard involved when the substances are given by inhalation. This is a rather important point, since quantitative inhalation studies are not particularly easy to carry out. Often a laboratory does not have adequate inhalation facilities to carry out such studies. Therefore it is possible to get some idea of relative hazard by using other routes of administration, provided one realizes that the absolute values obtained cannot be identical to those one would obtain if proper inhalation studies were carried out.

While this type of quantitative comparison of potencies can be very useful, it must be realized that quantal data say nothing about the severity of the haptic lesion. They merely indicate whether or not hepatic dysfunction has occurred. Table 4 summarizes the varying degrees of severity obtained for seven halogenaed hydrocarbons in dogs and mice when the amount of hydro-

Table 4. Severity of liver injury induced by minimal lethal doses of seven halogenated hydrocarbons, SGPT elevation being used as the index of hepatic dysfunction.[a]

Compound	SGPT (R-F units)	
	Dogs	Mice
Carbon tetrachloride	13,000	5,500
Chloroform	5,000	1,200
1,1,2-Trichloroethane	700	130
Dichloromethane	500	nil
Tetrachloroethylene	400	nil
1,1,1-Trichloroethane	350	65
Trichloroethylene	250	90

[a] Data obtained from Klaassen and Plaa (*3*, *4*).

carbon administered was quite large and in the lethal range (about an LD_{10}). One notes that the elevation in transaminase produced by carbon tetrachloride and chloroform is much greater than the elevations observed with the other hydrocarbons. This has been substantiated by morphologic assessment, and indeed the extent of the liver injury produced by carbon tetrachloride and chloroform with these doses is much greater than that produced with the other hydrocarbons included in the study.

While these results were obtained with substances that produced hepatic necrosis, one should not lose sight of the fact that the same principles can be used for measuring hyperbilirubinemia and cholestasis, α-Naphthylisothiocyanate (ANIT) produces cholestasis and hyperbilirubinemia in mice and rats (*6,7*). After having established normal bilirubin concentrations in a control population and establishing the upper limits of normalcy, one can also obtain quantal data for the presence or absence of hyperbilirubinemia. This is shown in Figure 2 for ANIT in mice; one can see that the production of hyperbilirubinemia follows a dose-related pattern and that the ED_{50} for production of hyperbilirubinemia is quite different from the LD_{50} in these animals.

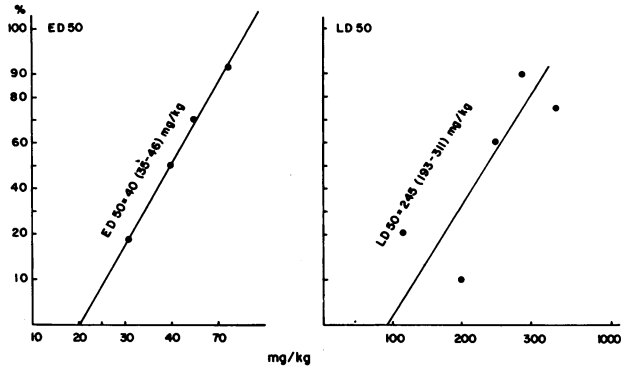

FIGURE 2. Dose-response curve for (left) hyperbilirubinemia and (right) lethality in mice 24 hr after orally administered ANIT. Data obtained from Becker and Plaa (*6*).

Quantitation of Other Abnormal Hepatic Biochemical Findings

The halogenated hydrocarbons like chloroform and carbon tetrachloride produce an increase in hepatic triglycerides which leads to the development of a fatty liver. Figure 3 shows the time course of this elevation in liver triglycerides for four halogenated hydrocarbons in rats (*8*). It can be seen that carbon tetrachloride causes a rapid accumulation of liver triglycerides and causes a more marked retention of triglycerides than the other compounds studied; 1,1,1-trichloroethane did not produce elevations in liver triglycerides. This elevation in liver triglycerides is also dose-dependent (Fig. 4). Such dose-response curves can be used for making quantitative comparisons among several halogenated hydrocarbons.

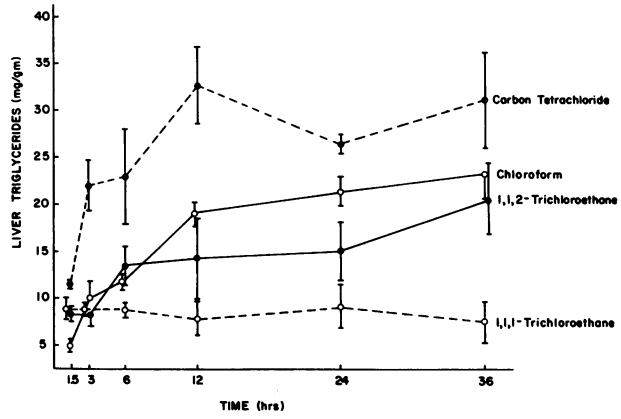

FIGURE 3. Effect of four halogenated hydrocarbons on hepatic triglyceride accumulation in rats. Agents were given intraperitoneally. Redrawn from Klaassen and Plaa (*8*) with permission of Pergamon Press.

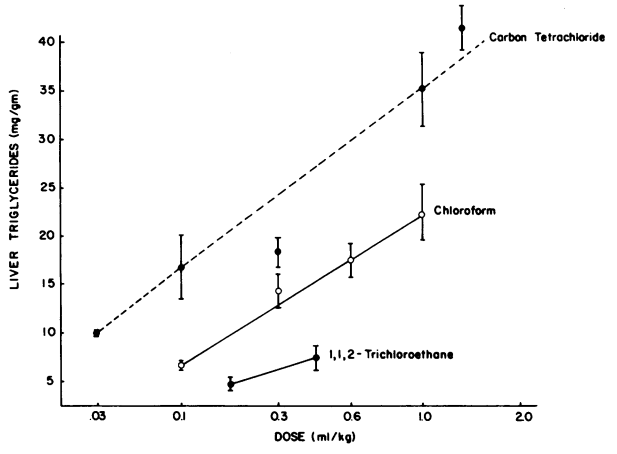

FIGURE 4. Dose-response curves for hepatic triglyceride accumulation for three halogenated hydrocarbons in rats. Agents were given intraperitioneally; triglycerides were measured 24 hr later. Redrawn from Klaassen and Plaa (*8*) with permission of Pergamon Press.

Unfortunately a number of reports in the literature which attempt to compare the relative properties of various halogenated hydrocarbons fail to realize that equitoxic doses have to be used in order to make such comparisons. In certain types of studies, like those which deal with mechanisms of actions, it is often necessary to compare compounds. In this type of situation it would seem essential that equitoxic doses, rather than equimolar doses, be employed. Quantitation of effects such as triglyceride accumulation can permit one to derive equitoxic doses.

Carbon tetrachloride is known to depress glucose-6-phosphatase activity in liver tissue. This enzyme is part of the endoplasmic reticulum and is measured in the microsomal fraction of liver homogenates. Figure 5 demonstrates the temporal pattern of the depression of glucose-6-phosphatase activity after exposure of rats to carbon tetrachloride (*8*). Such a depression in glucose-6-phosphatase activity is also a dose-related phenomenon and can be quantitated by determining dose-response curves (Fig. 6).

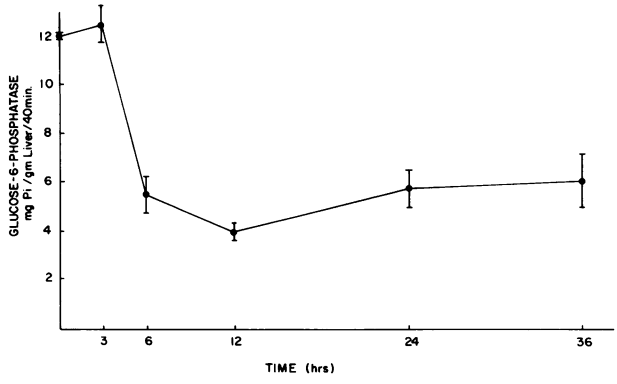

FIGURE 5. Effect of carbon tetrachloride, given intraperitoneally on hepatic glucose-6-phosphatase activity in rats. Redrawn from Klaassen and Plaa (*8*) with permission of Pergamon Press.

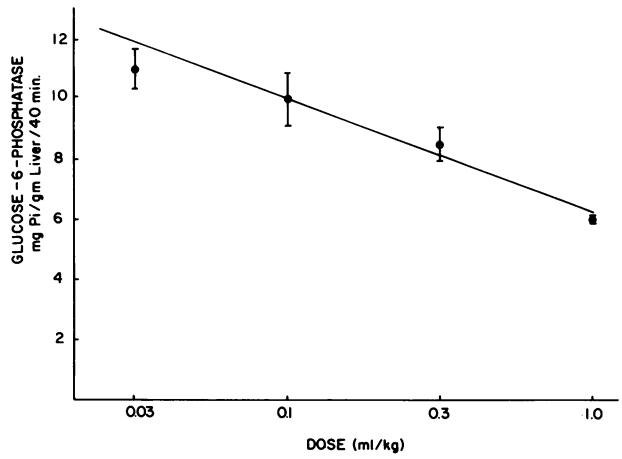

FIGURE 6. Dose-response curve for carbon tetrachloride on hepatic glucose-6-phosphatase activity in rats, 12 hr after intraperitoneal administration. Redrawn from Klaassen and Plaa (*8*) with permission of Pergamon Press.

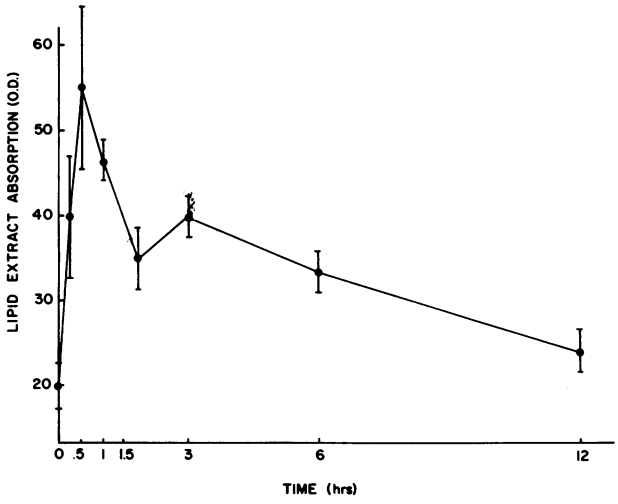

FIGURE 7. Effect of carbon tetrachloride, given intraperitoneally, on hepatic microsomal conjugated dienes in rats. Redrawn from Klaassen and Plaa (*8*) with permission of Pergamon Press.

Another known effect of carbon tetrachloride is the production of lipid peroxidation of microsomal lipids. This can be measured by following the formation of conjugated dienes in microsomal lipids. Figure 7 shows the temporal pattern of this lipid peroxidation following a single dose of carbon tetrachloride (*8*). This enhanced formation of conjugated dienes has also been demonstrated to be dose-related (Fig. 8).

The quantitative treatment of these various biochemical parameters of hepatic dysfunction can also be used to determine what types of tests are most likely to show alterations in interaction studies. In the rat for a 10-fold increase in dose (0.1 to 1.0 ml/kg) of carbon tetrachloride one finds that there is about a 4- to 5-fold difference in the elevation in serum transaminase values (*9*). From Figure 4 it is seen that there is about a 3-fold difference in hepatic triglyceride accumulation over the same dosage range. From Figure 6 it is seen that there is only about a 50% dif-

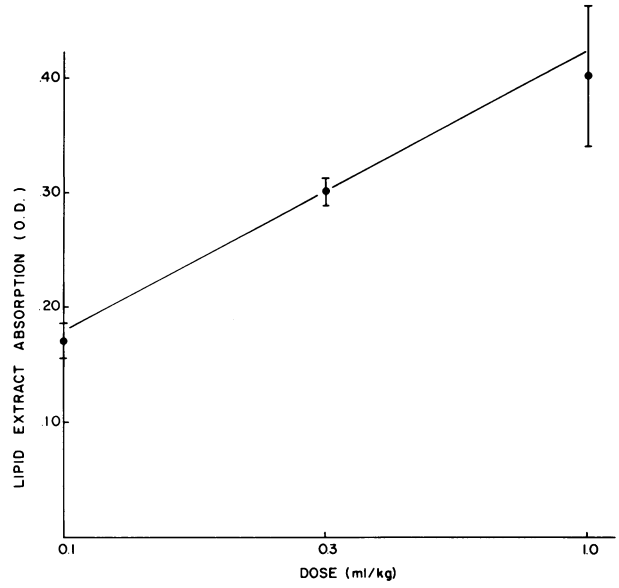

FIGURE 8. Dose-response curve for carbon tetrachloride on hepatic microsomal conjugated dienes in rats, 30 min after intraperitoneal administration. Redrawn from Klaassen and Plaa (*8*) with permission of Pergamon Press.

ference in the depression of hepatic glucose-6-phosphatase activity over this dosage range, and in Figure 8 it is seen that there is less than a 2-fold difference in the hepatic conjugated diene response. Therefore one would predict that relatively small differences in dosage will have a much more marked effect on transaminase activity, if this is the parameter being measured, than they would have on the presence of conjugated dienes or on the depression of hepatic glucose-6-phosphatase. This has been borne out in interaction studies carried out with ethanol, isopropanol, and carbon tetrachloride (*9*). The greatest effect of the interaction was seen when serum transaminase activity was the parameter measured and the least effective parameter appeared to be depression of hepatic glucose-6-phosphatase activity.

Quantitation in Chemical Interaction Studies

On the basis of industrial experience it was thought that ethanol could potentiate the liver injury produced by carbon tetrachloride or chloroform. In mice the conversion of prolongation of barbiturate sleeping time into quantal data was used to demonstrate the interaction between ethanol and chloroform (*10*). The number of animals exhibiting hepatic dysfunction when chloroform alone was given was considerably less than the number of animals showing hepatic dysfunction when ethanol was administered previously. The same type of potentiation was demonstrated by using BSP retention as the quantal parameter (*10*).

More recently, the potentiating effects of ethanol and isopropanol have been studies in the rat (*9*). This effect is shown in Figure 9, where elevation in serum transaminase activity is plotted against the pretreatment time. It can be seen that if ethanol was given 18 hr before carbon tetrachloride there was a significant increase in the transaminase elevation caused by a small dose (0.1 ml/kg) of carbon tetrachloride. Nonpretreated animals only exhibited an increased SGPT of about 150 units, whereas animals treated with ethanol 18 hr previously responded with 500 units of activity. However, with isopropanol the same small dose of carbon tetrachloride produced sufficient damage to elevate the transaminase activity to about 2400 units. This marked potentiation by isopropanol relative to that produced by ethanol has been verified by morphologic studies. The degree of the potentiation can be further seen when one compares the response of rats to 1.0 ml/kg of carbon tetrachloride in the nonpretreated animals; this 10-fold increase in carbon tetrachloride only produced about 450 units of SGPT activity whereas 1/10 of the dose given to animals pretreated with either ethanol or isopropanol yielded values of 500 and 2400 units, respectively. However, the

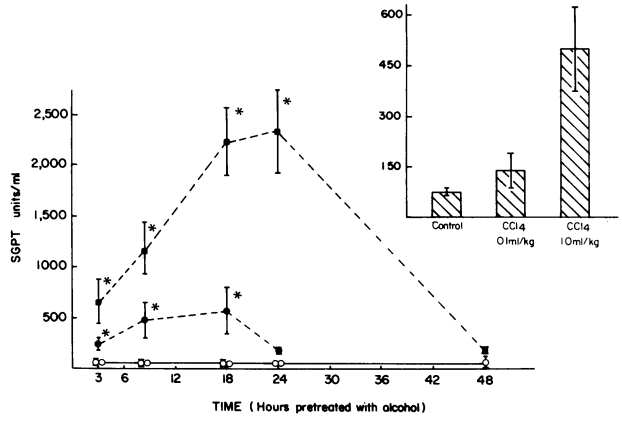

FIGURE 9. Effect of orally administered (•) ethanol and (■) isopropanol on carbon tetrachloride-induced elevation in serum transaminase activity, 24 hr after intraperitoneal administration of carbon tetrachloride to rats. The challenging dose of carbon tetrachloride was 0.1 ml/kg. Redrawn from Traiger and Plaa (*9*) with permission of Academic Press, Inc.

quantitative aspects shown in Figure 9 suggested that the two alcohols were not necessarily operating by the same mechanisms.

The marked quantitative difference between isopropanol and ethanol was investigated to try to unravel the mechanisms involved. It was established that, with isopropanol, its metabolic product, acetone, also could potentiate carbon tetrachloride liver injury (*11*). However with ethanol, its metabolic product, acetaldehyde, did not potentiate carbon tetrachloride. In other words, with isopropanol both the parent alcohol and its metabolic product appeared to be involved in the potentiation, whereas with ethanol only one substance was involved. By quantitative comparisons of the dose-response curves for isopropanol and acetone it was possible to determine the relative contribution of acetone in the isopropanol interaction (*11, 12*). This is demonstrated in Figure 10. It was calculated that about 80% of the isopropanol was converted to acetone in the rat. Therefore animals were subjected to varying doses of isopropanol to determine the dose-response relationship of this alcohol. Subsequently doses of acetone which would be equivalent to the amount converted *in vivo* were given to another series of rats and the dose-response curve determined. Figure 10 shows that the dose-response curves for acetone and isopropanol are virtually superimposable, suggesting that the major effect in the isopropanol interaction with carbon tetrachloride is due primarily to the formation of acetone. Once again quantitative assessment of hepatic dysfunction has been shown to be useful for determining the relative contribution of a pair of mixtures.

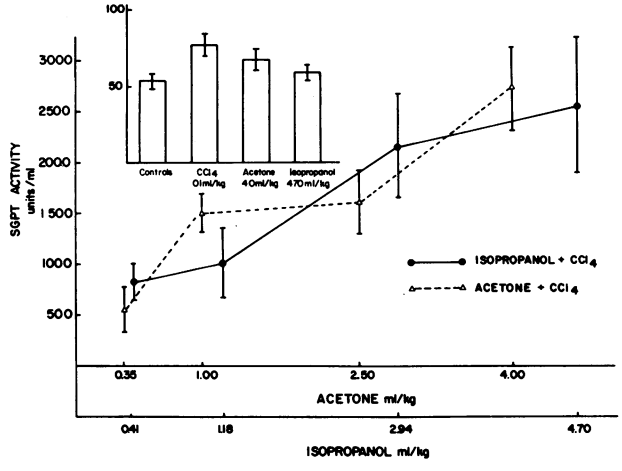

FIGURE 10. Dose-response curves for (△) acetone and (●) isopropanol potentiation of carbon tetrachloride-induced elevation in serum transaminase activity 24 hr after a challenging dose of carbon tetrachloride. Redrawn from Plaa and Traiger (*12*) with permission of S. Karger AG.

Quantitative Problems Involved in Low-Frequency Adverse Reactions

Cholestatic reactions that are seen with drugs in man occur with an extremely low frequency (*13*). While abnormal hepatic function can be demonstrated in large numbers of patients taking such drugs, only very few (1% or less) of individuals have sufficiently altered hepatic function to demonstrate the signs and symptoms of cholestasis and jaundice. Unfortunately these low-frequency reactions are not easily demonstrable in laboratory animals. Therefore some have suggested that our current toxicological testing methods are inadequate. Table 5 demonstrates the problem as it is presented to toxicologists. It should be remembered that the agents that produce cholestasis do so in a manner which appears to be unrelated to the dose.

Table 5. Number of animals required to show low frequency adverse reactions.[a]

Frequency, %	Number of animals to be tested
1	299
0.1	2,995
0.01	29,956
0.001	299,572

[a] Data obtained from Zbinden (*14*).

Therefore increasing the dose in an animal population does not necessarily uncover the cholestatic potential of a given drug. Zbinden (*14*) has calculated the number of animals which would be required to demonstrate an adverse reaction in animal populations if the frequency of this adverse reaction occurs with the same frequency found in humans. From Table 5 it can be seen that if the frequency in man is of the order of 1%, it would be necessary to test about 300 animals in order to have a 95% probability of getting one animal with the lesion. If the frequency of the adverse reaction in man is 0.1%, 300 animals would have to be tested to have a 95% probability of obtaining one reacting animal. If the frequency were 0.01% then 30,000 animals would have to be tested to yield one reacting animal

(95% probability). These calculations demonstrate that if the adverse reaction occurs in the animal population with the same frequency that it occurs in humans, it is virtually impossible to detect this type of change in the animals and also virtually impossible to collect a sufficient number of reacting animals to study the phenomenon. It is quite possible that the problem of drug-induced cholestasis as it is now known to occur with drugs is a demonstration of this low-frequency problem. This means that the mere increase in numbers of animals is not the solution to current toxicological testing procedures. This means that other types of animal models which respond with higher frequency have to be found or that other testing parameters which appear to correlate with cholestatic potential should be used as an index of cholestasis. Once again we see that the quantitative assessment of toxicity, this time frequency, plays an important role.

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